Infection, Genetics and Evolution 11 (2011) 912–916

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

TGFB1 and *IL8* gene polymorphisms and susceptibility to visceral leishmaniasis

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ARTICLE INFO

Article history:
Received 14 May 2010
Received in revised form 20 January 2011
Accepted 22 February 2011
Available online 2 March 2011

Keywords:
Visceral leishmaniasis
Cytokines
Gene polymorphisms
Disease susceptibility

ABSTRACT

Visceral leishmaniasis (VL) or Kala-azar is a serious protozoan infectious disease caused by an obligate intracellular parasite. Cytokines have a major role in determining progression and severity of clinical manifestations in VL. We investigated polymorphisms in the *TGFB1*and *IL8* genes, which are cytokines known to have a role in onset and severity of the disease. Polymorphisms at *TGFB1* −509 C/T and +869 T/C, and *IL8* −251 A/T were analyzed by a PCR-RFLP technique, in 198 patients with VL, 98 individuals with asymptomatic infection positive for a delayed-type hypersensitivity test (DTH+) and in 101 individuals with no evidence of infection (DTH−). The presence of the T allele in position −509 of the *TGFB1* gene conferred a two-fold risk to develop infection both when including those with clinical symptoms (DTH+ and VL, grouped) or when considering DTH+ only, respectively $p = 0.007$, OR = 1.9 [1.19–3.02] and $p = 0.012$, OR = 2.01 [1.17–3.79], when compared with DTH− individuals. In addition, occurrence of hemorrhage was associated with *TGFB1* −509 T allele. We suggest that the −509 T allele of the *TGFB1* gene, a cytokine with a biologically relevant role in the natural history of the disease, may contribute to overall susceptibility to infection by *Leishmania* and to severity of the clinical disease.

1. Introduction

Visceral leishmaniasis (VL) or Kala-azar (black fever) is a chronic disease caused by an obligate intracellular protozoan parasite, that targets internal organs like liver, spleen, bone marrow, and lymph nodes. The disease is caused by different *Leishmania* species, but in Brazil mainly by *Leishmania chagasi*, where 3000–5000 new cases are reported each year (Berman, 2006). The disease is predominantly pediatric (Malla and Mahajan, 2006) with a large spectrum of clinical manifestations ranging from asymptomatic to a severe visceral involvement. Clinical disease is caused by parasites infecting the internal organs. In addition to effects due to inherent characteristics of the infecting parasite, the immune response against *Leishmania* can be substantially modified by factors such as the nutritional status, age, and genetic background of the host (Caldas et al., 2002; Desjeux, 2004)

In most subjects, infection progresses to spontaneous cure. In these cases, symptoms of overt disease are absent and infection can be evidenced only by positivity to leishmanin in an intradermal delayed-type hypersensitivity test (DTH). Thus, infection is usually self-limited but eventually progresses to the more severe form, presenting fever, profound cachexia, hepatosplenomegaly, and even severe bleeding, leading to death if left untreated (Blackwell et al., 1997; Caldas et al., 2005).

The clear dichotomy of the immune response present in murine isogenic models of leishmaniasis is not a feature of human disease. In man, cytokine profiles change according to disease progression. Individuals with the active form of the disease exhibit increased IL-8, IFN-γ, TNF-α, IL-6, IL-12, and TGF-β1 in an inactive state, but normal levels of anti-inflammatory cytokines such as IL-10 (Barral-Netto et al., 1992; Caldas et al., 2005; Peruhype-Magalhães et al., 2006). It is the balance between these cytokines that will impact upon prognosis (van der Poll et al., 1995; Blackwell, 1998; Kaye et al., 2004; Kumar et al., 2009). After completing treatment with antimonials, TGF-β1, IL-10, as well as IFN-γ and IL-12 plasma

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doi:10.1016/j.meegid.2011.02.014

levels will remain high for a certain period (Caldas et al., 2005). Furthermore, asymptomatic DTH+ individuals show a cytokine profile similar to non-infected individuals, which will be distinguished from non-infected controls in cell cultures after short-term stimuli with specific *Leishmania* antigens, where they respond with the typical mixed Th1/Th2 response. (Caldas et al., 2005).

The role of TGF-β1 in VL has been recognized since 1992 (Barral-Netto et al., 1992). TGF-β1 is directly involved in monocyte/macrophage activation, inhibiting the production of nitric oxide and oxygen free radicals that exert microbicidal activity against the parasite. It also inhibits production of the pro-inflammatory cytokine IFN-γ by lymphocytes, directing the immune response to a Th2 profile (Bogdan and Nathan, 1993) and contributing to progression to a more severe form of VL in the infected patient. Caldas et al. (2005) studying *Leishmania*-infected individuals in São Luiz, Maranhão, Brazil, showed that high TGF-β1 plasma levels are associated with the active form of the VL (Caldas et al., 2005). Increased TGF-β1 is also strikingly observed in bone marrow obtained from patients with VL (Gantt et al., 2003). In addition, Saha et al. (2007), studying peripheral blood mononuclear cells of patients with Kala-azar and post Kala-azar, from two endemic regions in India, showed increased production of TGF-β1 in response to specific antigen in patients with the active form of the disease.

Plasma IL-8 levels are also increased in VL patients, as shown in subjects from an endemic area in Belo Horizonte, Brazil (Peruhype-Magalhães et al., 2006). In Bangladesh, patients with clinical and sub-clinical Kala-azar also show elevated IL-8 plasma levels (Bern et al., 2006). A member of the family of CXC chemokines, IL-8 is a major player in amplifying inflammatory processes (Harada et al., 1994) and a strong chemoattractant for neutrophils. Furthermore, neutrophils infected with *Leishmania* increase IL-8 production further increasing recruitment of immune cells (van Zandbergen et al., 2004). IL-6 primarily produced by macrophages and T cells, has an important role in macrophage activation, proliferation, and differentiation (Castell et al., 1989). Increased IL-6 plasma levels have also been found in both VL patients with active disease and in asymptomatic DTH+ individuals showing high rates of cure (van der Poll et al., 1995; Caldas et al., 2005; Ansari et al., 2006). In addition, the *IL6* gene has been associated with mucosal leishmaniasis in a previous study (Castellucci et al., 2006).

TGF-β1, IL-8, IL-10, IL-12, IL-6, IFN-γ, TNF and GM-CSF are some of the factors from an extended list that participate in the pathogenesis of VL, ultimately resulting in an overall cytokine profile that will help determine the clinical characteristics and the severity of the disease in infected individuals. Polymorphic variants in the genes that code for these cytokines could help explain the diversity in the clinical presentation of the disease and are candidates for studies aiming to assess genetic susceptibility to VL. Polymorphisms in the *IFNG*, *TNF*, *IL4*, and *IL10* genes have been described previously (Farouk et al., 2010; Hulkkonen et al., 2001; Karplus et al., 2002; Jeronimo et al., 2007). There are two widely studied functional polymorphisms in the *TGFB1* gene. One of them is the −509 C/T polymorphism in the *TGFB1* promoter region. T allele carriers exhibit higher circulating TGF-β1 levels (Grainger et al., 1999). The exchange of C to T creates a YY1 activator consensus sequence associated with transcription regulation (Shrivastava and Calame, 1994). Another polymorphism in the exonic region in position +869 (T/C) defines an aminoacid substitution (leucine for proline) resulting in increased TGF-β1 levels (Wong et al., 2003). A polymorphism in position −251 (A/T), in the promoter region of the *IL8* gene, has been associated with higher circulating levels of this cytokine (Lurje et al., 2008) and with increased transcription measured by a reporter assay (Lee et al., 2005). Finally, the best studied polymorphism in the IL6 gene, located in the promoter region (−174 G/C) impacts upon IL-6 serum levels (Belluco et al., 2003) We conducted this study to determine if these polymorphisms in *TGFB1*, *IL8*, and *IL6* genes are involved in susceptibility to VL and if an association can be established with relevant clinical data and cytokine plasma levels.

2. Methods

2.1. Brazilian population sample

A total of 397 individuals (aged 0–66 years), namely 101 DTH− (65 females and 39 males), 98 DTH+ (45 females and 53 males), and 198 VL (79 females and 109 males), the majority children (n = 177) aged 0–17 years, treated with pentavalent antimonium, from designated endemic areas in the states of Piauí and Maranhão, were included in this study. About 40% of the children with VL showed some complications, such as bleeding and other infections. In the group of adult patients, 19% tested positive for HIV. All individuals were diagnosed based on clinical, parasitological and immunological parameters. DTH− individuals were considered as controls for comparison with VL patients and DTH+ individuals (asymptomatic group), which, in spite of contact with the parasite, do not develop active disease. Blood samples were obtained from all studied groups after giving written informed consent according to recommendations of the Internal Review Board Ethics Committees from the participating centers in Maranhão and Piauí.

2.2. Isolation of genomic DNA

Genomic DNA was obtained from 5 mL of EDTA-treated blood mononuclear cells using phenol/chloroform following standard procedures (Gustincich et al., 1991).

2.3. Analysis of gene polymorphisms

Single nucleotide polymorphisms (SNPs) at different positions of the IL6, *IL8* and *TGFB1* genes, namely IL6 −174 G/C, *IL8* −251 A/T, *TGFB1* −509 C/T and *TGFB1* +869 A/T, were typed by PCR-RFLP (Pulleyn et al., 2001; Wong et al., 2003; Lurje et al., 2008; Sainz et al., 2008). Pairs of primers used as well restriction enzymes and PCR product sizes are described in Table 1. PCR was performed in a final volume of 25 μL containing 50 ng of genomic DNA, 40 μM of dNTP and 0.2U of Taq polymerase, 1.5 mM $MgCl_2$ and 0.25 μM of each primer. PCR conditions included an initial denaturation step at 95 °C for 5 min followed by 35 cycles of amplification at a specified temperature and a final cycle of extension at 72 °C for 7 min. A volume of 10 μL of the PCR products was digested with the corresponding restriction enzymes. The fragments were identified by agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide and visualized under UV light.

2.4. Statistical analysis

Genotype and allele frequencies were calculated by direct gene counting. For comparison among VL, DTH+ and DTH− individuals we applied the Chi-square test with odds ratio (OR) considering a 95% confidence interval [CI]. Statistical comparison was also carried out by Fisher's exact test whenever a value in the contingency table was below five, using Graph Pad Prism 5.0 software. All genotype frequencies were tested for deviation from Hardy–Weinberg equilibrium (HWE). The power of the sample size to detect statistically significant differences, considering the type I error as 0.05 and a relative risk of 2.5, in the SNP frequencies among all the groups studied (VL, DTH+ and DTH−) was calculated separately for each SNP using PS (Power and Simple Size

Table 1
Primer sequences, restriction enzymes and digestion products obtained by PCR-RFLP.

Gene/SNP	rs n°	Allele wt/ var		Region	Primers	T°C PCR	RE	Digestion products (bp)
IL-6							*Nla III*	
−174 G/C	18007951	G	C	Promoter	F: TGACTTCAGCTTTACTCTTGT R: CTGATTGGAAACCTTATTAAG	51	2U	wt 122/45/31 var 167/31
IL-8							*MfeI*	
−251 A/T	4973	A	T	Promoter	F:TTGTTCTAACACCTGCCACTCT R: TGTGAGACTCAGGTTTGCC'	61	1U	wt 141/80 var 221
TGFB1							*Dde I*	
−509C/T	1800469	C	T	Promoter	F: GGGGACAGTAAATGTATGĠ R:TGAGACACAGGGGAGCC	56	2U	wt 144/50/18 var 197/50/18
TGFB1							*MspA1*	
+869T/C	1982073	T	C	Exon 1	F: TTCAAGACCACCCACCTTCT R:TCGCGGGTGCTGTTGTACA	60	2U	wt 285 var 273/12

SNP: single nucleotide polymorphism; rs: reference SNP; wt: wide type allele; var: variant allele; RE: restriction enzyme; U: units; bp: base pairs.

Calculations, v.2.1.30 software at http//biostat.mc.vanderbilt.edu/twiki/bin/view/Main/Power Sample Size). A PS over 70% was considered as adequate for our sample size.The four SNPs studied were submitted to Chi-square or Fisher's test to identify associations with the following clinical and laboratory parameters, age, dyspnea, edema, kidney failure, splenomegaly, duration of disease, jaundice, hemorrhage, bacterial infection, AST (aspartate aminotransaminase) and ALT (alanine aminotransaminase) levels (under or above 60 U/L), platelet count (under or above 50,000/mm^3), and cytokine plasma levels (altered values considered above 25 pg/mL). SNPs were considered as independent variables and clinical and laboratory parameters were considered as dependent variables for application in univariate logistic regression models using STATA 8.2 software. Spearman correlation analysis was done using data from 111/198 VL patients that had their TGF-β1 and IL-8 plasma levels determined at Fundação Osvaldo Cruz, Salvador, Bahia. TGF-β1 levels showed a high variability ranging from 10.8 to 6000 pg/mL (71.2 ± 726 pg/mL). Only 15 patients had normal levels <25 pg/mL. IL-8 plasma levels varied from 17.5 to 2910 pg/mL (182.1 ± 333.9 pg/mL), but almost all patients except 2, had increased levels of this cytokine.

3. Results

3.1. Polymorphisms

The genotype distribution for all loci was in Hardy–Weinberg equilibrium.

Initially to determine if the size sample would be capable to detect correctly the polymorphisms, a power test (PS) was applied. For the *IL8* −251 A/T, *TGFB1* −509 C/T and *TGFB1* +869 T/A polymorphisms, calculated PS were 90%, 73% and 88%, respectively, indicating that the number of samples included in the study was adequate to detect correctly the frequency of these polymorphisms. We initially proposed to study also the *IL6* −174 G/C SNP, but did not pursue the analysis further due to the low frequency of the rare allele in our sample, and, therefore a very low PS of only 10%.

The presence of the T allele (TT + TC vs CC) in position −509 of the *TGFB1* gene conferred a 1.9 fold risk to develop disease, when VL and DTH+ individuals were compared as one group (infected) to DTH− controls ($p = 0.007$, OR = 1.90 [1.19–3.02]). The increase TT + TC genotype was confirmed when VL or DTH+ groups were individually compared with DTH− individuals (Table 2). Of note,

Table 2
Genotype and allele frequencies of *TGFB1* −509 C/T polymorphism in the visceral leishmaniasis, positive delayed-type hypersensitivity and negative delayed-type hypersensitivity groups.

	Individuals in contact with *Leishmania*		Controls	Statistical values for comparison		
TGFB1 −509 C/T	VL *n* = 196 (%)	DTH+ *n* = 101 (%)	DTH− *n* = 98 (%)	χ^2	*p* Value	OR (95%CI)
Genotype						
CC	63 (32)	29 (29)	45 (46)			
CT	106 (54)	45 (44)	41 (42)	12.74	0.013	
TT	27 (14)	27 (27)	12 (12)			
Genotype comparison TT and TC vs CC						
LV vs DTH +				0.40	0.545	0.95 (0.5–1.44)
LV vs DTH+ and DTH−				1.12	0.300	1.25 (0.83–1.9)
DTH+ vs DTH−				6.40	0.012	2.11 (1.17–3.79)
LV and DTH+ vs DTH−				7.26	0.007	1.90 (1.19–3.02)
LV vs DTH−				5.33	0.020	1.80 (1.09–2.95)
Allele						
C	232 (59)	103 (51)	131 (67)	10.34	0.006	
T	160 (41)	99 (49)	65 (33)			
Allele comparison C vs T						
LV vs DTH +				3.64	0.056	1.40 (0.99–1.96)
LV vs DTH+ and DTH−				0.01	0.910	1.02 (0.76–1.35)
DTH+ vs DTH−				10.31	0.001	1.94 (1.29–2.01)
LV and DTH+ vs DTH−				6.64	0.010	1.56 (1.11–2.19)
LV vs DTH−				3.24	0.070	0.72 (0.50–1.03)

VL: visceral leishmaniasis; DTH+: positive delayed-type hypersensitivity; DTH−: negative delayed-type hypersensitivity; *n*: number individuals; $\aleph^2$: qui square; OR: odds ratio; CI: confidence interval

Table 3
Genotype and allele frequencies of *TGFB1* +869 T/C polymorphism in the visceral leishmaniasis, positive delayed-type hypersensitivity and negative delayed-type hypersensitivity groups.

TGFB1 +869 T/C	VL $n=172$ (%)	DTH+ $n=92$ (%)	DTH− $n=85$ (%)	χ^2	p Value
Genotype					
CC	38 (22)	24 (26)	21 (25)		
CT	96 (56)	48 (52)	40 (47)	2.35	0.671
TT	38 (22)	20 (22)	24 (28)		
Allele					
C	172 (50)	96 (52)	82 (48)	0.55	0.758
T	172(50)	88 (48)	88 (52)		

VL: visceral leishmaniasis; DTH+: positive delayed-type hypersensitivity; DTH−: negative delayed-type hypersensitivity; *n*: number individuals; χ^2: qui square.

Table 4
Genotype and allele frequencies of *IL8* −251 A/T polymorphism in the visceral leishmaniasis, positive delayed-type hypersensitivity and negative delayed-type hypersensitivity groups.

IL8 −251 A/T	VL $n=184$ (%)	DTH+ $n=96$ (%)	DTH− $n=97$ (%)	χ^2	p Value
Genotype					
AA	45 (25)	15 (16)	22 (23)		
TA	80 (43)	48 (50)	46 (47)	3.24	0.518
TT	59 (32)	33 (34)	29 (30)		
Allele					
A	170 (46)	78 (41)	90 (46)	1.84	0.398
T	198 (54)	114 (59)	104 (54)		

VL: visceral leishmaniasis; DTH+: positive delayed-type hypersensitivity; DTH−: negative delayed-type hypersensitivity; *n*: number individuals; χ^2: qui square.

the number of TT homozygotes was increased only in the DTH+ individuals but not in VL or DTH− individuals.

The individual allele distribution confirmed differences between VL subjects and DTH− controls or between DTH+ individuals compared to DTH− controls. The remaining SNPs showed similar distribution in the three groups analyzed (Tables 3 and 4).

There was no correlation of *TGFB1* and *IL8* polymorphisms with the corresponding plasma cytokine levels from VL patients. Of all laboratory and clinical parameters analyzed, only hemorrhagic events were associated with the presence T allele at *TGFB1* −509 C/T. Out of 147 patients with complete follow-up, 30 presented bleeding events. Though present on patients without the symptom (74/117, 63.2%), T allele was overrepresented in the group with hemorrhagic events (25/30, 83.4%) with a calculated Chi-square of 4.38 and $p = 0.036$. The same level of significance was observed in the logistic regression analysis.

4. Discussion

The factors that lead individuals living in the same endemic area, city, neighborhood or even the same house, to respond differently to leishmaniasis infection, presenting different clinical symptoms and responses to treatment have been only partially defined, but available data suggest that the genetic background contributes to the outcome of VL emphasizing the importance of identifying the genes involved in the physiopathology of the disease. In this study, *TGFB1* and *IL8* gene polymorphisms were chosen due to the previously described functional role of these cytokines in the pathogenesis of the disease.

The individuals analyzed are natives from two neighboring but different endemic regions. Separate analysis of the samples showed the same allele distribution for all genes studied, thus we chose to merge the samples in the final analysis. Though the study would benefit from a replication study either in an independent sample or in an extension from the same endemic regions, we could not for the moment, obtain further samples, due to the inherent difficulties of doing field research in these regions.

No differences were observed in the allele distribution of the *IL8* −251 A/T SNP when the three groups of individuals were compared. In addition, in the VL group of patients, no relationship between higher IL-8 levels and the *IL8* SNP was observed. Approximately 20% of the patients exhibited IL-8 plasma levels above 200 pg/mL irrespective of the genotype, and practically all patients showed increased levels of this cytokine (data not shown). There are several other known *IL8* gene polymorphisms, not analyzed in our study. However, they are found in strong linkage disequilibrium with *IL8* −251 A/T (Hull et al., 2004) indicating that a role for polymorphisms in the *IL8* gene is not to be expected.

The presence of the T variant in position −509, but not of the +869 T/C polymorphism of the *TGFB1* gene, was significantly increased in VL and DTH+ individuals compared to DTH− controls. This study was not designed for prospective analysis, and, thus, initial TGF-β1 serum levels are not known, and their impact, or of the gene polymorphism upon the initial infection by the parasite cannot be ascertained. However, *L. chagasi* promastigotes interfere directly in the levels of TGF-β1 surrounding the infected macrophages through activation of cystein proteinases, especially cathepsin B, that release the active molecule from latent TGF-β1 (Gantt et al., 2003) leading to local immune downregulation, which serves locally as an escape mechanism by the parasite in the infected macrophages (Rodrigues et al., 1998; Omer et al., 2000). Thus, in a similar way to the proposed role of SLC11A1 (former NRAMP1) variants associated with VL and DTH+ status (Mohamed et al., 2003), lower microbicidal activity and a dampened immune innate response would contribute to conversion of the susceptible individuals to the infected status.

No association between T allele carriage and serum TGF-β1 levels was evidenced (data not shown). TGF-β1 is released in an inactive form bound to latency-associated peptide, is deposited in the extracellular matrix bound to a second protein latency TGF-β binding protein and must be released from both to be activated. Furthermore, once activated, turnover is very rapid. Thus, the higher level of circulating TGF-β1 in VL is the end result of many different events occurring within the infected organism that could mask the effect of the gene variant (Gantt et al., 2003).

Intriguingly, a significant relation between hemorrhagic episodes and the presence of the T allele was evidenced. We suggest an increase in local production of TGF-β1 in the liver might add to the extensive fibrosis observed leading to decrease in thrombopoietin production (Gleizes et al., 1997). The ensuing lowered platelet count may tip the balance in favor of increased hemorrhage in some of the patients with severe VL.

In conclusion, our results suggest that the −509 T allele of the *TGFB1* gene, a cytokine with a biologically relevant role in the natural history of the disease, may contribute to overall susceptibility to infection by *Leishmania* and to severity of the clinical disease.

Acknowledgements

This study received financial support from the Brazilian National Research Council (CNPq). A.F.F. was supported by a CAPES fellowship and A.C.G., J.K., A.B., M.B-N., C.H.N.C. are recipients of personal achievement grants from CNPq.

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